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Hypertonicity induced apoptosis in HL-60 cells in the presence of intracellular potassium

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Abstract Cell shrinkage is a hallmark of apoptosis. Potassium efflux, which is involved in cell shrinkage, has been previously described as an essential event of apoptosis. This study was designed to address the importance of potassium efflux in hypertonicity (450 mOsm and 600 mOsm) induced apoptosis. We initiated apoptosis in HL-60 cells in hypertonic medium consisting of either high concentrations of NaCl, mannitol or KCl. Apoptotic activity was evaluated based on the DNA content of the cells, annexin-V staining and calcium content. Apoptosis was initiated in hypertonic conditions consisting of high intracellular K^+. We demonstrate that apoptosis can occur in the presence of high intracellular potassium contrary to previous predictions.

Keywords Cell death · Apoptosis · Volume regulation · Annexin-V · DNA fragmentation · Hypertonicity · HL-60

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Abbreviations

RVD	regulatory volume decrease
RVI	regulatory volume increase
AVD	apoptotic volume decrease

Introduction

The process that drives cell shrinkage during apoptosis is known as apoptotic volume decrease (AVD) [1]. Cell volume decrease is however not restricted to apoptotic cells, it occurs in some cell types in response to changes in osmolarity of the extracellular environment. Most cells when placed in hypotonic solution, initially swell passively and then recover their volume by a process known as regulatory volume decrease (RVD) [2]. In both RVD and AVD K^+ efflux, accompanied by Cl^-, results in cell volume to decrease [3–6]. Blocking of potassium channels with quinine inhibits RVD in human promyoletic leukemia (HL-60) and monocytic leukemia (THP-1) cells [7], as well as AVD and the subsequent caspase increase in human leukemic monocyte lymphoma (U937) and rat phaeochromocytoma (PC 12) cells [8].

Further evidence that apoptosis requires K^+ loss and/or the resulting cell shrinkage comes from the observation that reduction of net K^+ efflux by suspending lymphocytes in high K^+ medium reduces DNA fragmentation [9]. In agreement with this hypothesis, potassium channel blocker quinine blocks staurosporine-induced cell death in rat neuroblastoma (NG108-15) cells [8]. Blocking of K^+ channels participating in AVD has been shown to apparently block apoptosis in different cell types [8, 10]. All together the above-mentioned experiments indicate that lowering of intracellular potassium is essential for apoptosis at least in some cell types.

Previous studies have examined the changes of HL-60 volume in anisotonic solutions for relatively short time periods [11]. In this study we examined the longer-term effects of alteration of cell volume. We show that HL-60 cells are also susceptible to hypertonicity and undergo apoptosis as shown before in lymphoid cells [12]. Inducing hypertonicity in the presence of high Na^+, K^+ or mannitol, we were able to put the cells in environments where certain ion efflux would be virtually impossible. In HL-60 cells we detected hypertonicity induced apoptosis sans potassium efflux. Our results indicate that potassium efflux is a key regulator of apoptosis but not necessary for it to occur.

Materials and methods

Cell culture

HL-60 cells (American Type Culture Collection, VA) were cultured with RPMI-1640, 10% FBS (Gibco, Grand Island, NY) and 1% Penstrep (Grand Island, NY). Cells were incubated at 37°C and 5% CO_2. The cell culture concentration was approximately 1 million cells/ml at the time of experimentation. There was no detectable variation in the cell cycle profile of untreated cultures between experiments. Cell cultures consisted of 49.1% (SEM ± 1.5) of cells in G0/G1, 14.1% (SEM ± 1.1) in G2 and 36.8% (SEM ± 1.7) in S phase of the cell cycle. The cell cycle profiles were assessed using the PI labeled DNA on the flowcytometer.

Solutions used

Cells were exposed to isotonic and anisotonic conditions by mixing solutions of different osmolarities 1:1 to the RPMI-1640 cell culture medium. For cell culture, isotonic medium (osmolarity 288 to 293 mOsm) consisted of solution 'Iso' composed of 109 mM NaCl, 4 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM Hepes (Sigma, St. Louis, MO) and 23.7 mM $NaHCO_3$ dissolved in distilled deionized H_2O (ddH_2O), this solution was then mixed 1:1 with cell culture medium. Volume measurements of cells exposed to isotonic medium were obtained in solution 'Iso'. High hypertonic medium (601 to 608 mOsm) was obtained by either increasing the concentration of NaCl or KCl or by the addition of Mannitol (Sigma, St. Louis, MO). High-KCl hypertonic medium was obtained by mixing solution 'High K-1' 1:1 with cell culture medium. High K-1 solution had all the same constituents of solution Iso, except that it had no NaCl and 463 mM KCl. Similarly, high NaCl or mannitol hypertonic medium was obtained from a solution containing 450 mM NaCl or 600 mM mannitol respectively. Medium hypertonic medium (443–449 mOsm) were similarly made using solutions containing 306 mM NaCl or 314 mM KCl or 283 mM mannitol depending on the kind of hypertonicity desired mixed 1:1 with cell culture medium. Volume measurements were conducted in solutions of their respective osmolarities, instead of mixing hypertonic solutions 1:1 with cell culture medium, they were mixed with solution Iso. Hypotonic media (approximately 150 mOsm) was obtained by 1:1 dilution with ddH_2O containing 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM Hepes. All solutions were adjusted to pH 7.4 at 25°C.

Cell volume measurements

Volume measurements were obtained using a 256-channel Elzone electronic particle analyzer (Orifice diameter 95 μm). The instrument was calibrated using beads of diameter 5, 10 and 20 μm. The final concentration of cells during measurements was maintained at 8.6×10^3 cells/ml. A minimum of 10000 cells/sample were analyzed. The solutions (as described in the section *solutions used*) were maintained at 37°C.

Analysis using flow cytometer

Apoptosis detection by sub-Go/G1 DNA contents using flow cytometry

Cells were fixed in 70% ethanol for 24 h at 2–4°C. They were then washed with PBS, incubated with RNase (1 mg/ml, Sigma) for 30 min, and stained with propidium iodide (PI), 20 μg/ml. After a time lapse of 10 min, the cells were immediately analyzed by flow cytometry. The cells were gated according to their size and DNA content (for example see Fig. 3(E) and (F)). Intact cells with less than Go/G1 DNA content were classified as apoptotic.

Annexin-V staining, cell viability detection and calcium measurements using flow cytometry

A minimum of 10000 cells/sample was analyzed using a FACS Vantage sorter/ flow cytometer (BD Immunology, Palo Alto, CA) fitted with an argon-ion laser emitting 360 nm and 488 nm wavelength. Annexin V-FITC (apoptosis detection kit, CalBiochem, USA) signal was detected at 518 ± 40 nm. Propidium iodide (2 μg/ml) was added prior to analysis for gating apoptotic cells from necrotic cells. Cell viability assessment involved monitoring both the size (determined using forward and side scatter measurements) and PI content of the cell. Intracellular calcium concentration was measured with Indo-1 AM (Sigma, 2 mg/ml) as described earlier [13].

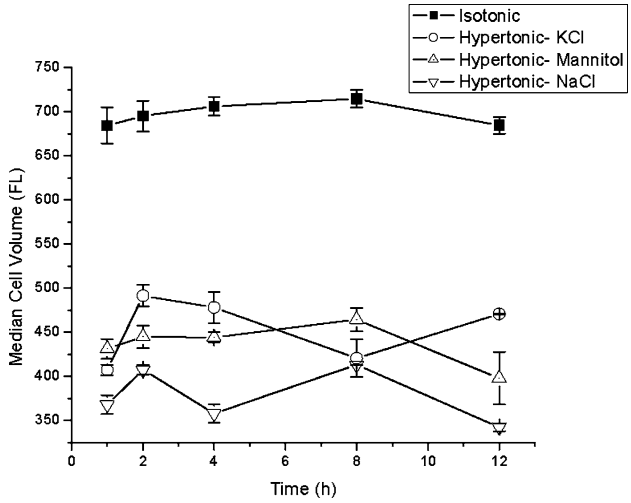

Fig. 1 Median volume of cells as a function of time in culture. Points represent the average of measurements of the median cell volume of the HL-60 cell population obtained from three independent experiments. Cells were exposed to hypertonicity of 600 mOsm. Error bars represent standard error of the mean (SEM). Paired *t*-tests and ANOVA on ranks followed by Dunnett post hoc test with significance level of $p < 0.05$ when comparing control to that of the hypertonic conditions revealed significant difference at all time points measured

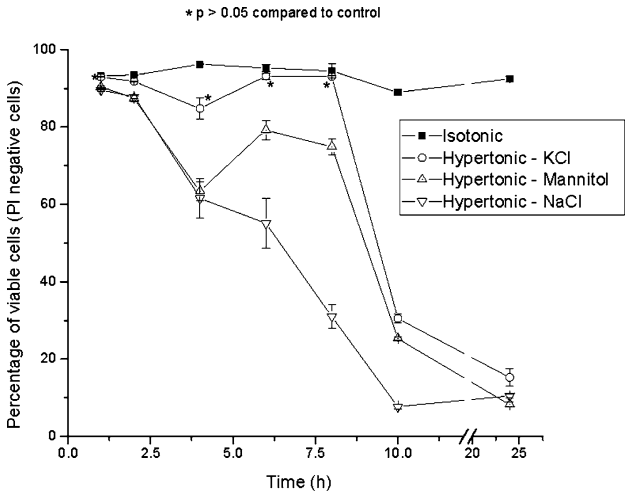

Fig. 2 Cell viability as assessed through propidium iodide (PI) exclusion using flow cytometry. Hypertonic conditions (as in Fig. 1) reduced the cell viability of HL-60 cells. All points other than indicated (with '*') were found to be statistically significant. Error bars represent SEM

Results

Similar persistent cell volume reduction induced by hypertonicity irrespective of the ionic constituent

Hypertonic environment of approximately 600 mOsm induced pronounced cell volume decrease. Previously our group has shown that this process takes a few minutes to complete and HL-60 cells remain shrunken for at least 30 min [2]. In this study, exposure of cells to hypertonicity constituted by high KCl, mannitol or NaCl in the medium, showed an induction of persistent volume decrease lasting up to 12 h (Fig. 1).

Loss of cell viability in hypertonic condition

The percentage of propidium iodide (PI) permissive cells in culture, used as an indicator of non-viable cells, significantly increased from 4 h post exposure to hypertonicity (600 mOsm) and continued for the next 21 h that we measured (Fig. 2). Loss of cell viability was slower in hypertonic cell cultures consisting of high KCl compared to other osmolytes.

Apoptosis is induced by hypertonicity even in the presence of high KCl

The above-mentioned loss of cell viability could be a result of either apoptosis or necrosis. We used PI to detect DNA fragmentation on fixed cells and PI-Annexin V staining on live cells, both these methods have been extensively used and established as reliable indicators of apoptosis [14–16]. DNA fragmentation is one of the final stages of apoptosis. Prominent increase in percentage of cells with fragmented DNA was seen in all cell populations treated with hypertonicity (Fig. 3(A), (B) and (E)). Apoptosis induced at 600 mOsm was more pronounced and faster compared to the apoptosis induced at 450 mOsm. Like other hypertonic conditions, high KCl hypertonic medium induced pronounced apoptosis by 8 h at 600 mOsm and by 12 h at 450 mOsm. At both 450 mOsm and 600 mOsm, percentage of apoptotic cells over the time course of 12 h appeared higher in medium with high NaCl than other osmolytes.

Annexin-V staining of externalized phosphatidylserine in cells that exclude PI is an early, caspase dependent, marker of apoptosis [15, 17]. We measured the percentage of Annexin-V positive cells in a viable cell population treated with 600 mOsm hypertonicity (Fig. 3(C), (D) and (F)). A significant increase of Annexin-V positive cells was seen in the first 2 h in all hypertonic conditions. Cells treated with high KCl hypertonicity induced a slower rise in the percentage of apoptotic cells when compared to the other hypertonic conditions between 4 to 8 h (Fig. 3(D)).

Calcium levels in high hypertonic condition

Intracellular calcium is known to be a key mediator in the induction and progression of apoptosis. In our experiments, intracellular calcium levels were measured using Indo-1 simultaneously during Annexin-V apoptosis detection in cells exposed to 600 mOsm hypertonicity (Fig. 4). We measured intracellular calcium in apoptotic (annexin-V positive but PI

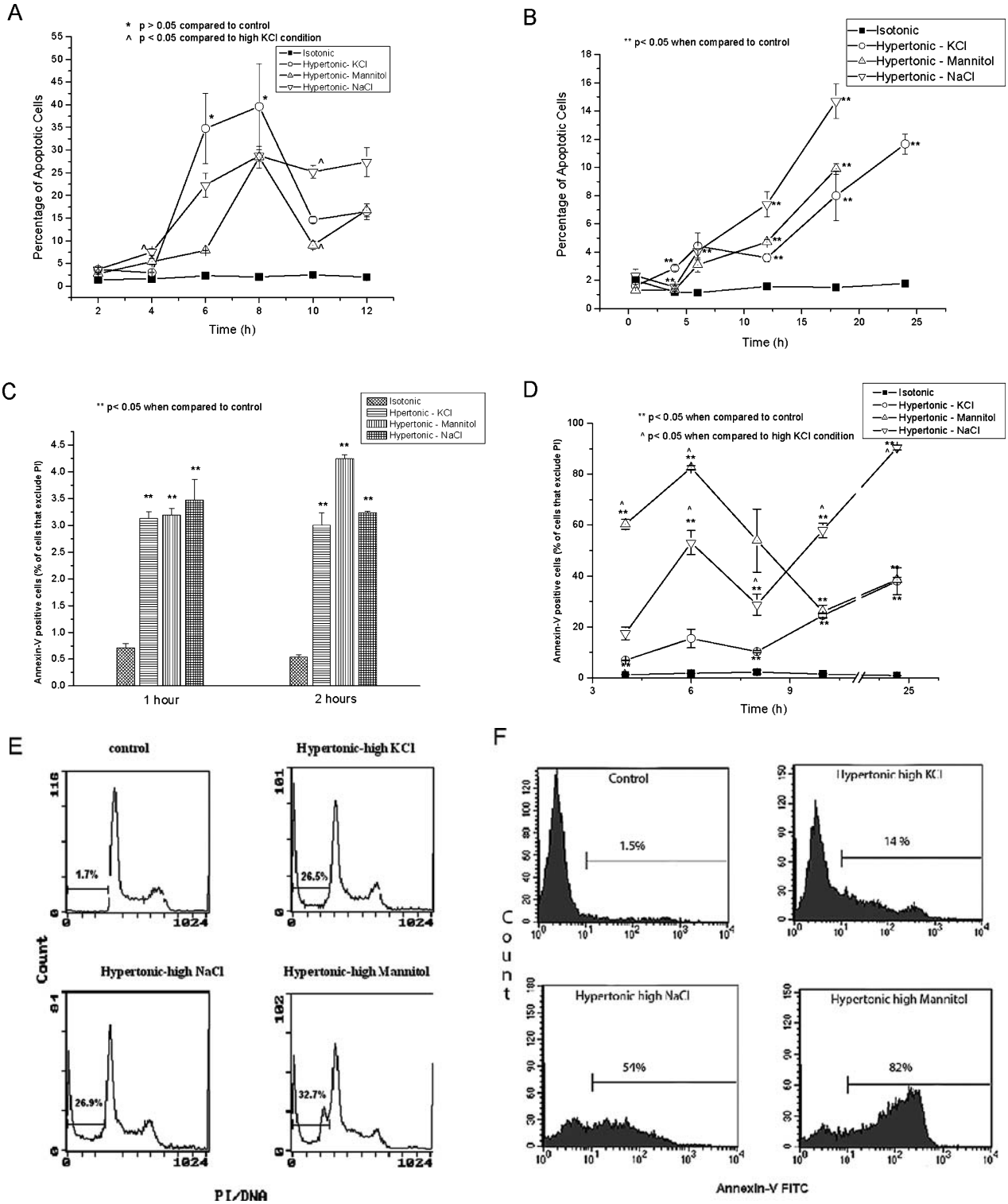

Fig. 3 Detection of apoptosis using flowcytometry. Percentage of sub-Go/G1 (apoptotic) cells is depicted in HL-60 cells when exposed to 600 mOsm solutions (Panel A) and 450 mOsm solutions (B). Annexin-V staining was monitored overtime in HL-60 cells exposed to 600 mOsm hypertonicity (C and D). Panel E shows the representative DNA fragmentation profiles under control and hypertonic conditions (600 mOsm). Bars show the gates used to measure the percentage of apoptotic cells (defined as those with hypodiploid DNA content) and were determined from the isotonic cell culture DNA profile). Panel F shows the profiles of Annexin-V labeling in cell cultures under control and hypertonic conditions (600 mOsm)

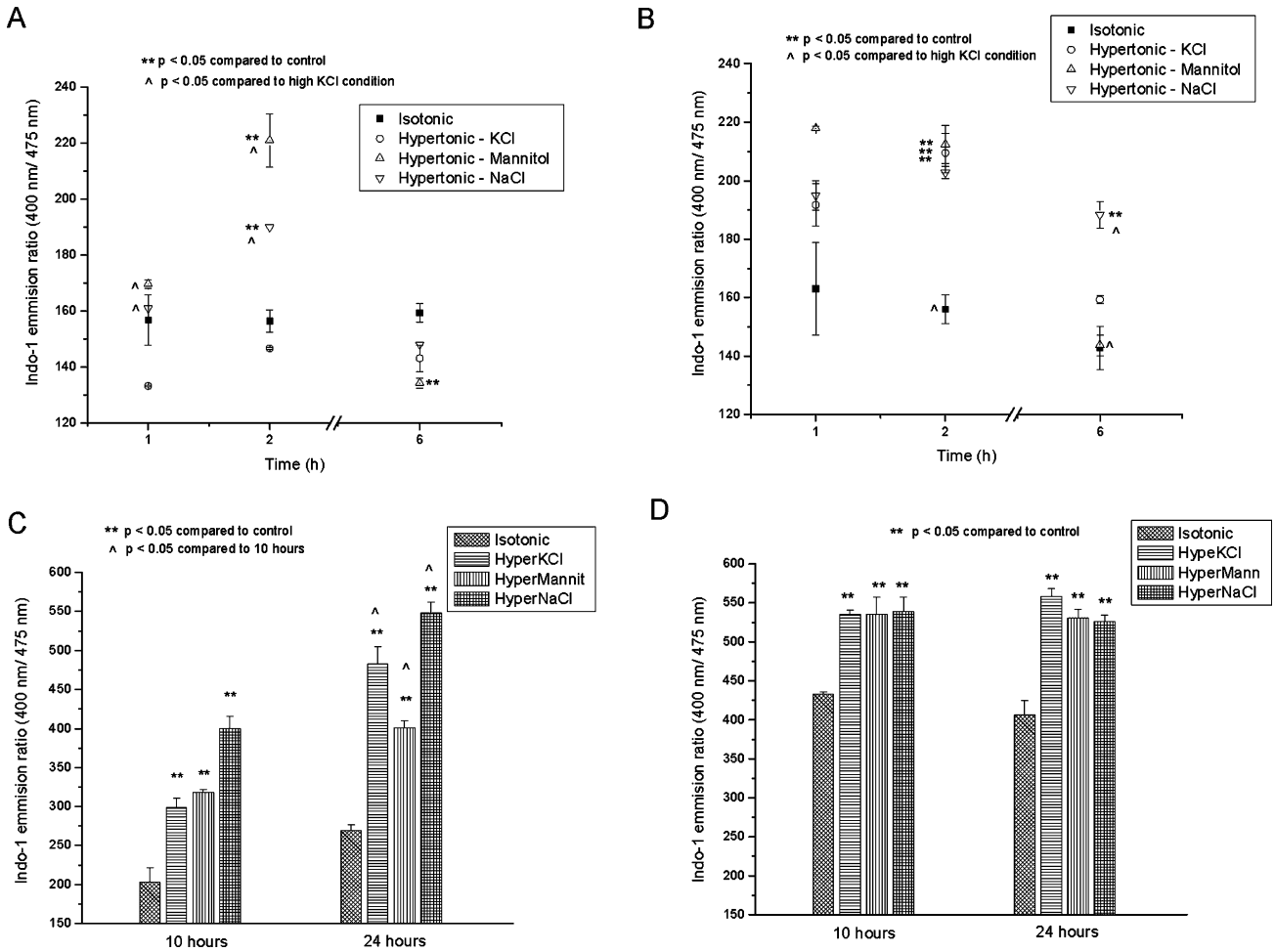

Fig. 4 Monitoring of intracellular free calcium in Annexin-V positive and negative cells. Intracellular calcium was measured using Indo-1 AM as described in experimental methods exclusively in PI negative cells. Levels of intracellular free calcium in Annexin-V negative cells (A) increased at early time points in all hypertonic conditions, except in high KCl medium. All hypertonic conditions increased intracellular calcium in Annexin-V positive cells (B). At later time points, both Annexin-V negative (C) and positive (D) cells show a significant increase of intracellular calcium under hypertonic conditions when compared to the control

negative cells, Fig. 4(B) and (D)) and non-apoptotic cells (annexin-V negative and PI negative cells, Fig. 4(A) and (C)). Apoptotic cells under hypertonic conditions showed an initial rise in intracellular calcium in the second hour. The higher levels of intracellular calcium persisted in all the hypertonic conditions at 10 and 24 h. Even in cells that were neither annexin-V positive nor PI positive (apparently non apoptotic) showed high levels of intracellular calcium at 10 h and had increased even further at the 24th hour post induction of hypertonicity (Fig. 4(C)).

Lack of pronounced apoptosis in hypotonic condition

HL-60 cells when exposed to hypotonicity of 150 mOsm underwent regulatory volume decrease (RVD) and recovered to attain their isotonic volume that remained stable for hours (Fig. 5(A)). The percentage of apoptotic cells estimated by DNA-fragmentation showed a negligible increase at 4 and 12 h when compared to the extent of apoptosis induced in hypertonic conditions (Fig. 5(B)). The percentage of apoptotic cells in a population treated with hypotonicity did not exceed 4%.

Discussion

Exposure of HL-60 cells for 1 h or longer to hypertonic media under conditions where volume regulation is absent (hypertonic, lack of RVI) leads to apoptosis. These results agree with earlier work indicating a close relationship between volume regulation and apoptosis [18–23], and add support to the concept that changes in volume itself are able to initiate signals that lead to caspase-3 activation and DNA fragmentation. The induction of apoptosis in the presence of high potassium shows that maintenance of intracellular potassium

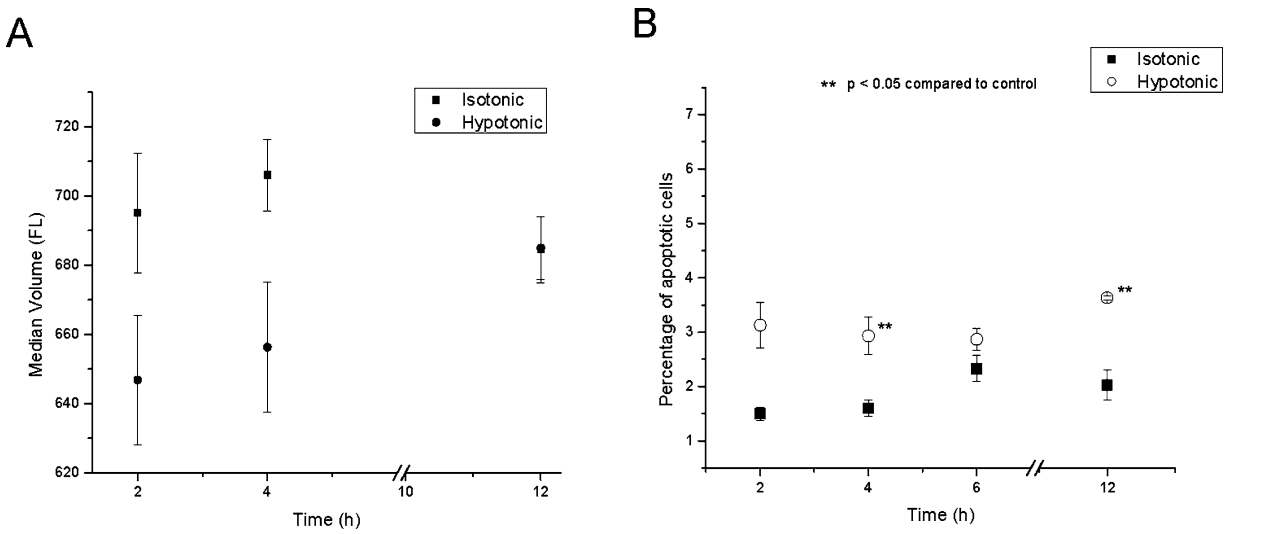

Fig. 5 Monitoring of apoptosis under hypotonic conditions. Hypotonicity alone does not induce significant apoptosis. Hypotonicity did not lead to any long term significant changes of cell volume. (A) DNA fragmentation analysis did not detect any increases in the proportion of apoptotic cells as seen under hypertonic conditions (B)

is not sufficient to prevent caspase-dependent apoptosis in HL-60 cells.

Mannitol based hypertonicity induces apoptosis in HL-60 cells

Previous studies have shown that HL-60 cells lack rapid RVI [2]. Our results (Fig. 1, upright triangles) show that in a medium made hypertonic (2 times isotonic) with mannitol the volume decreases to about 65% of normal after 1 h, and does not change significantly for the next 11 h. The extent of shrinkage is consistent with our laboratory's previous observation of an osmotically inactive volume in neutrophils of about 260 femtoliters [2].

During this time there is a continuous increase in both annexin-V positive cells (Fig. 3(C)) and in DNA fragmentation (Fig. 3(B)), indicating that hypertonicity alone causes apoptosis in HL-60 cells, as it does in lymphoid cells [12]. Surprisingly, no further volume decrease (AVD) takes place, which indicates that these apoptotic changes are not accompanied by efflux of K^+ and Cl^- through the channels that mediate AVD. Although 2 times more osmolyte must leave the cell in the hypertonic medium in order to produce a given volume decrease, cell water loss should increase both intracellular $[K^+]$ and $[Cl^-]$, thus increasing the driving force for K^+ and Cl^- efflux. This in turn should increase the rate of net KCl efflux in proportion to the increase in tonicity, provided that the AVD channels are open. The absence of further volume decrease after the initial shrinkage due to water loss, therefore, indicates that at least one of the channels (K^+ or Cl^-) does not open, possibly because of inhibition caused by the shrunken state of the cells, and/or that other mechanisms (e.g. replacement of intracellular K^+ by Na^+) cause dissipation of the $[K^+]$ gradient, reducing the driving force for apoptotic K^+ loss.

Induction of apoptosis in the presence of high potassium

We induced apoptosis in the presence of hypertonicity consisting of high extracellular potassium or sodium. Hypertonicity consisting of potassium was slower in inducing apoptosis than other hypertonic conditions. Since extracellular $[K^+]$ is much higher than intracellular $[K^+]$ in the high KCl medium, K^+ efflux is virtually impossible unless some other unknown mechanisms of potassium efflux are involved. Apoptotic thymocytes and T cell hybridoma cells have been shown to leak intracellular K^+ [24], even this nature of leak or K^+ efflux through Ca^{++} activated K^+ channels [25] would be difficult against the concentration gradient. In spite of this, apoptosis was possible in a caspase dependent manner as inferred due to increased PS externalization and DNA fragmentation (Fig. 3(A–D)).

Previous studies have suggested that the decrease in internal potassium concentration normally seen during AVD in isotonic media causes activation of enzymes which lead to DNA fragmentation [1]. The evidence for apoptotic changes in hypertonic media might be explained by the fact that K^+ does not completely inhibit these enzymes [1]. Another possible mechanism which might explain apoptosis in the presence of high cytosolic $[K^+]$ is that increased uptake of K^+ into mitochondria leads to mitochondrial swelling and cytochrome *c* release [1]. It is also likely that K^+-selective channels involved in AVD cause replacement of intracellular K^+ by Na^+ in the shrunken, apoptotic cells.

In the presence of high extracellular [Na^+], as in our NaCl hypertonic medium, intracellular [K^+] is expected to increase due to cell volume shrinkage. Na^+ efflux through Na^+/K^+ ATPase influences AVD in HL-60 cells [20]. Our results suggest role of Na^+ efflux in apoptosis is distinct from K^+ efflux, since preventing Na^+ efflux in a high NaCl environment did not decrease the rate of apoptosis but rather enhanced it. The slower pace of apoptosis in high KCl medium might be due to the known protection provided by high intracellular potassium. Interestingly treating HL-60 cells with clofilium, a potassium channel blocker, induces apoptosis [26]. The induction of apoptosis by clofilium is presumably due to other pharmacological properties of clofilium that are not directly related to blocking of potassium channels [27]. However, the fact that apoptosis can occur when potassium channels are blocked and our results suggest that while potassium efflux is a regulator of the pace of apoptosis, it is not necessary for induction of apoptosis.

Rise in cytosolic calcium in annexin-V negative cells in hypertonic conditions

Persistent rise in cytosolic [Ca^{++}] is known to trigger apoptosis and is related to the potassium efflux in AVD [20, 24]. As expected annexin-V positive cells showed higher cytosolic free Ca^{++} (Fig. 4(B) and (D)). Annexin-V negative cells, presumably not fully committed to apoptosis, increased cytosolic free Ca^{++} in hypertonic conditions by 10 h (Fig. 4(A) and (C)). This increase indicates a calcium dependent mechanism of apoptosis due to hypertonicity irrespective of the constituent. This also supports the hypothesis that the apoptotic stimulus in hypertonic medium is cell shrinkage and the ionic constituent of the cell is a mediator of the rate of apoptosis. Loss of intracellular [K^+] also takes place due to hypotonicity during RVD. Thus cells exposed to hypotonicity after completion of RVD have less intracellular [K^+] than cells in isotonic condition. Cells exposed to hypotonicity show only marginal increase in apoptosis (Fig. 5(B)). Hypotonicity alone is thus not an effective stimulus for the induction of apoptosis in HL-60 cells.

Our results are apparently contradictory to previous predictions from known models of potassium efflux regulated apoptosis; few studies have been done regarding apoptosis induced by hypertonicity or cell shrinkage. Entirely different apoptotic pathways might be involved in hypertonicity induced apoptosis compared to apoptosis induced by various other agents. Cell volume changes not only cause ionic changes in the cell but also cytoskeletal changes [2]. Moreover, changes in macromolecular crowding due to alterations in cell volume [28] might alter protein structure [29] and thereby activate signaling pathways leading to apoptosis. Since HL-60 cells are a cultured cell model for the promyelocytic stage in neutrophil differentiation, it is notable that shrinkage of neutrophils was shown to increase phosphorylation and activation of mitogen-activated protein kinase p38 [30]. Inhibition of this kinase prevented shrinkage-induced shedding of L-selectin from the neutrophil surface. It has also been reported that p38 is involved in signaling of stress-activated neutrophil apoptosis [31]. Thus, the factors of changes in ion composition and ionic strength, macromolecular crowding, and consequent changes in protein structure and activity may all contribute to the triggering of apoptosis in a hypertonic environment.

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